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Original Article

Comparison of Quinn's Advantage fertilization medium and tissue culture medium 199 for *in vitro* maturation of oocytes

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ABSTRACT

Objective: The purpose of the study was to compare the Quinn's Advantage fertilization medium (Q1) and the tissue culture medium 199 (TCM199) for *in vitro* maturation (IVM) of oocytes and ammonium production during IVM.
Materials and methods: The immature murine oocytes were randomly added into Q1 and TCM199. Ammonium concentrations were measured at the start and after 18 hours of IVM, and the mature oocytes were fertilized and cultured into blastocysts. The blastocysts were then stained for inner cell mass (ICM) and trophectoderm.
Results: The maturation rate was higher in Q1 than in TCM199 (85.7% vs. 76.6%, $p = 0.024$). The fertilization and blastocyst rates were slightly higher in Q1, but not significant. Differential staining of the blastocysts showed slightly higher ICM ratio in the blastocysts derived from Q1. Mean ammonium concentrations in Q1 and TCM199 at Time 0 were 184.9 and 339.2 μg/dL, respectively ($p = 0.05$), and after 18 hours of IVM were 268.7 and 443.6 μg/dL, respectively ($p = 0.045$). Addition of ammonium chloride into Q1 adversely affects IVM.
Conclusion: Q1 is superior to TCM199 in terms of oocyte maturation, which may be due to lower ammonium concentration.

Introduction

Immature oocyte retrieval followed by *in vitro* maturation (IVM) is an attractive alternative to *in vitro fertilization* (IVF) [1,2]. Because few or no injections of gonadotropins are needed, IVM reduces the patient's cost and suffering and avoids the side effects associated with ovarian stimulation. Despite recent progresses, the implantation rate of IVM is still lower than that of traditional IVF [3]. The inferior outcome of human IVM was thought to be primarily due to abnormalities of cytoplasmic maturation in *in vitro*-matured oocytes.

The maturation system is a vital component of IVM. Many strategies have been designed to improve the outcome of IVM, such as supplementation of serum, gonadotropins, and growth factors [1], human chorionic gonadotropin priming [4], follicle-stimulating hormone priming [3,5] before oocytes retrieval, and coculture [6]. It has been shown that the addition of glucose and amino acids in simple and complex media [e.g., tissue culture medium 199 (TCM199)] during bovine IVM can support embryonic development [7]. Zheng et al showed that the choice of amino acids in monkey IVM system affected the competence of oocyte maturation [8]. Amino acids are commonly added to the embryo culture media to enhance embryonic development, but their requirement for IVM is not fully understood.

Supplementation of embryo culture media with amino acids has been shown to benefit embryonic development in many mammalian species, including humans [9]. The effects of amino acids on embryonic development vary with regard to different amino acids

Conflicts of interest: The authors have no conflicts of interest to declare.

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and the stages of the embryos [10]. However, spontaneous amino acid breakdown, mainly glutamine, generates ammonium, which in turn induces aberrant blastocyst development and alters fetal development [11]. In contrast to the commonly used TCM199, Quinn's Advantage fertilization medium (Q1; SAGE, Trumbull, CT, USA) contains heat-stable alanyl-glutamine rather than glutamine. Therefore, we conducted a study to compare the outcomes of IVM with the two media, and ammonium production during IVM.

Materials and methods

The mice used in the study were FVB/NJNarl mice (National Laboratory Animal Center, Taipei, Taiwan). The experiment was approved by the Institutional Animal Care and Use Committee. The chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA) unless stated otherwise. The experiment was repeated six times.

IVM of oocytes

Immature oocytes were collected from 3- to 5-week-old female mice, 48 hours after intraperitoneal injection with 5 IU pregnant mare serum gonadotropin. Immature oocytes with cumulus cells were collected by teasing the ovarian follicles. Only cumulus–oocyte complexes that comprised oocytes with homogeneous cytoplasm and more than three layers of cumulus cells were used for the study.

The maturation medium consisted of Q1 or TCM199, plus 75 mIU/mL *human menopausal* gonadotropin (Merional; IBSA, Lamone, Switzerland), 0.2 mM pyruvate, and 10% fetal bovine serum (FBS). The immature oocytes were randomly added into Q1 and TCM199, and were incubated in groups of 10 at 37°C in an atmosphere of 5% CO_2 in air. Two blank media, namely, Q1-based and TCM199-based maturation media without oocytes, were incubated at the same time. Ammonium concentrations in the media were measured at the start and after 18 hours of IVM culture.

After 18 hours of IVM, the oocytes were assessed for maturity. Only oocytes that displayed a distinct first polar body were classified as in metaphase II (MII) and were considered further for fertilization and culture.

IVF

Spermatozoa were retrieved from the cauda epididymis of 5- to 7-week-old male mice. The spermatozoa were dispersed in the human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA) containing 5 mg/mL FBS, diluted to a concentration of 1×10^6/mL. The MII oocytes were incubated with the spermatozoa for 4 hours. The oocytes were then washed to eliminate excess spermatozoa and cultured overnight in a drop of mKSOM (Speciality Media Inc., Phillipsburg, NJ, USA) covered with mineral oil. The next morning, the number of two-cell embryos was counted, and the embryos were transferred to fresh droplets of mKSOM under mineral oil and cultured to blastocysts. The maturation rates, fertilization rates, and embryo development were recorded.

Differential staining of blastocysts

For the differential staining of trophectoderm (TE) and inner cell mass (ICM), blastocysts were incubated in 0.05% pronase solution in mKSOM for 5 minutes at 37°C to remove the zona pellucida. The blastocysts were washed in mKSOM and incubated in 0.5% 2,4,6-trinitrobenzene sulfonic acid solution for 10 minutes at 4°C in the dark, followed by a wash in mKSOM. The blastocysts were incubated at room temperature for 40 minutes in the dark in a stain solution, which contained 1 μg/mL bisbenzimide (Hoechst 33258) and 1 μg/mL propidium iodide in mKSOM. The stained blastocysts were washed again in mKSOM before fixation in 4% formalin solution. They were then stored in the dark at 4°C until observation. A single-layered image was obtained by applying gentle pressure on the blastocysts. The blastocysts were observed under a fluorescence microscope (Olympus AH3; Tokyo, Japan) equipped with an ultraviolet filter. The nuclei of ICM appeared blue and the nuclei of TE appeared pink.

Addition of ammonium chloride

To assess the effects of ammonium on IVM, ammonium was added into Q1. In the first part of the experiment, the mean ammonium concentration in Q1 at Time 0 was 184.9 ± 5.1 μg/dL; 180 μg/dL (Group 2) and 540 μg/dL (Group 3) ammonium chloride (Sigma) were added into Q1 to make ammonium concentrations approximately two and four times that of Q1 only (Group 1; i.e., 360 and 720 μg/dL). The immature oocytes were now added into the three media. After 18 hours of IVM, the mature oocytes were fertilized, and cultured to blastocysts. The experiment was repeated six times.

Ammonium concentrations in the maturation media were determined in duplicates with the enzymatic method and read on a spectrophotometer (model COBAS INTEGRA 800; Roche Diagnostics, Berlin, Germany) set at 340 nm. The intra-assay coefficient of variation from five samples was 4.8%.

Statistical analysis

Values were expressed as mean ± standard deviation. Ammonium concentrations were compared using the Mann–Whitney *U* test. Comparison of percentages between groups was carried out using the χ^2 test or one-way analysis of variance, as appropriate. A p value <0.05 was taken to be significant. Analyses were carried out using the SPSS statistical package, version 14.0 (SPSS Inc., Chicago, IL, USA).

Results

At Time 0, ammonium concentrations in Q1 and the TCM199 were 184.9 ± 5.1 and 339.2 ± 7.6 μg/dL, respectively ($p = 0.05$). After 18 hours of IVM, ammonium concentrations rose slightly in Q1 and TCM199 (both $p = 0.05$) to 268.7 ± 13.4 and 443.6 ± 15.5 μg/dL, respectively. The ammonium concentration after 18 hours was lower in Q1 than in TCM199 ($p = 0.045$). At the same time, ammonium concentrations in the blank Q1 and in the blank TCM199 without oocytes were 247.1 ± 22.0 and 457.0 ± 12.0 μg/dL, respectively. Ammonium concentrations in the same medium with and without oocytes were not significantly different.

The outcomes of IVM are shown in Table 1. The maturation rate was higher in Q1 than in TCM199. The fertilization rate and development to blastocysts were slightly higher in Q1, but not significant.

Table 1
Outcomes of IVM in TCM199 and Q1.

	TCM199	Q1	*p*
No. of oocytes	210	210	
MII oocytes	161 (76.6%)	180 (85.7%)	0.024
Fertilization	105 (65.2%)	128 (71.1%)	0.24
Morula	32 (30.5%)	45 (35.2%)	0.48
Blastocyst	16 (15.2%)	23 (18.0%)	0.6

IVM = *in vitro* maturation; MII = metaphase II.

The results of differential staining of blastocysts are shown in Table 2. There were no significant differences in total cell numbers and cell numbers of ICM and TE of the blastocysts derived from the two media. However, the ICM ratio was slightly higher in the blastocysts derived from Q1.

In the second part of the experiment in which ammonium chloride was added into Q1, mean ammonium concentrations at the start and after 18 hours of IVM in the three groups were 142.1 ± 1.9, 324.7 ± 12.6, 685.4 ± 11.6 μg/dL and 199.7 ± 3.7, 373.3 ± 5.2, 728.5 ± 1.6 μg/dL, respectively. The results of IVM in the three groups are shown in Table 3. The maturation rate, fertilization rate, and development to morulae and blastocysts in Group 1 were higher than those in Groups 2 and 3, and ammonium had a dose-dependent adverse effect on oocyte maturation.

Discussion

Although originally designed for bovine IVM, TCM199 is still used in some centers for human IVM [3,12–14]. No previous studies have compared the outcomes of IVM in TCM199 and Q1. Our study shows that the ammonium concentration was lower in Q1, and the oocyte maturation rate was slightly higher in Q1 than in TCM199. The fertilization rates and embryo development were slightly higher in Q1, but not significant. TCM199 contains all essential and nonessential amino acids, and is supplemented with vitamins. Q1 was modified from the HTF medium, which was first described by Quinn et al [15]. Modifications include low phosphate and addition of citrate, lactate, ethylenediaminetetraacetic acid, selected nonessential amino acids, taurine, and alanyl-glutamine (product insert, Table 4).

Certain amino acids are beneficial for embryo development [9,16] and IVM [7,17]. Supplementation of nonessential and essential amino acids in the bovine oocyte maturation media was associated with enhanced developmental frequencies, increased blastocyst cell number, and elevated oocyte maternal messenger RNA levels [17]. However, spontaneous breakdown of amino acids, especially glutamine, produced ammonium [16,18]. Instead of glutamine in TCM199, Q1 contains a heat-stable derivative of glutamine, alanyl-glutamine. Replacing glutamine with alanyl-glutamine reduced the accumulation of ammonium and resulted in significantly lower ammonium levels [11]. The present study showed that ammonium had a dose-dependent adverse effect on oocyte maturation. Addition of ammonium into Q1 to make its concentration similar to that in TCM199 also impaired IVM. Therefore, we speculate that the inferiority of TCM199 is due to a higher ammonium concentration.

This is the first study to show that ammonium was produced at high concentrations in TCM199. The ammonium concentrations increased slightly after 18 hours of IVM in both media. Ammonium concentrations in the same medium with and without oocytes were not significantly different, indicating that ammonium resulted mostly from the medium *per se*, rather than from the oocytes. Addition of ammonium into Q1 to make its concentration similar to that in TCM199 significantly reduced maturation and fertilization rate, and blastocyst development, but TCM199 only produced lower maturation rate than Q1, implying that TCM199 partially overcame the adverse effects of ammonium. Because the embryos were moved to mKSOM on the next day after fertilization, they were no longer affected by the ammonium in the maturation media. The adverse effects of ammonium on IVM are probably time and dose dependent. Yuan and Krisher showed that ammonium at concentrations ≥ 2 mM decreased porcine oocyte maturation, but embryo development was not affected until an ammonium concentration of ≥20 mM [19]. Gardner and Lane showed that ammonium production during embryo culture increased linearly with time, and its levels increased to potentially inhibitory levels after 18 hours [16]. In human IVM, the oocytes take 24–48 hours to mature. Therefore, the inhibitory effect of ammonium may be more pronounced in human IVM.

Ammonium is present in follicular fluid and its concentrations are highest in the smallest follicles and decrease as follicles grow [20]. The mean ammonium concentration in preovulatory follicular fluid is 38.87 μM (67.0 μg/dL) [21], which is much lower than the concentrations in freshly prepared maturation media and after 18 hours of IVM in our study. Few studies have investigated the effects of ammonium on IVM. Hammon et al showed that exposing bovine oocytes to 29–356 μM (50–613.8 μg/dL) of ammonium during IVM did not adversely influence embryonic development [20]. However, they did not investigate the effects of ammonium on oocyte maturation. The present study is compatible with their study in that development to blastocysts was unaffected, but we found that the maturation rate was affected by ammonium.

The mechanism of the detrimental effects of ammonium on IVM may be twofold. Ammonium reduced *in vitro* growth and metabolism of granulosa cells, and impaired their ability to support IVM of oocytes [22]. Second, exposure to ammonium may decrease

Table 2
Blastocyst cell numbers in different media.

	TCM199	Q1	*p*
Total cell number	83.1 ± 2.9	83.0 ± 3.2	0.749
ICM	17.0 ± 0.9	20.0 ± 1.1	0.969
TE cells	66.1 ± 2.1	63.0 ± 2.2	0.651
ICM ratios	0.20	0.23	0.026

Values are mean ± standard deviation.
ICM = inner cell mass; TE = trophectoderm.

Table 3
Outcomes of IVM in Q1 and addition of ammonium.

	Group 1	*p* (Group 1 vs. Group 2)	Group 2	*p* (Group 2 vs. Group 3)	Group 3
No. of oocytes	180		180		180
MII oocytes	156 (86.7%)	0.001	116 (64.4%)	0.037	98 (54.4%)
Fertilization	106 (67.9%)	0.006	58 (50%)	0.958	50 (51.0%)
Morula	52 (49.1%)	0.009	12 (20.7%)	0.087	2 (4.0%)
Blastocyst	28 (26.4%)	0.003	4 (6.9%)	0.211	0

Group 1, Q1; Group 2, Q1 + 180 μg/dL ammonium chloride; Group 3, Q1 + 540 μg/dL ammonium chloride.
IVM = *in vitro* maturation; MII = metaphase II.

Table 4
Comparison of components of the TCM199 and Q1.

Components	TCM199	Q1
Essential amino acids	+	−
Nonessential amino acids	+	L-Asparagine, L-aspartic acid, glycine, L-proline, L-serine, taurine
Alanyl-glutamine	−	+
Inorganic salts (e.g., NaCl, KCl, $MgSO_4$, $NaHCO_3$, potassium phosphate)	+	+
Glucose	+	+
Pyruvate	+	+
Lactate	−	+
Sodium citrate	−	+
EDTA	−	+
Vitamins	+	−

EDTA = ethylenediaminetetraacetic acid.

intracellular pH [11], and intracellular pH regulatory mechanisms are inactive in immature oocytes [23].

In conclusion, the study showed that Q1 was superior to TCM199 for IVM. Ammonium was produced in the maturation media and had adverse effects on IVM. Utilizing media or maturation systems that produce less ammonium may improve the outcome of IVM.

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